Pharmacology of Phosphoinositides, Regulators of Multiple Cellular Functions

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Abstract: Inositol phospholipids represent a small fraction of the phospholipids present in all cellular membranes with remarkable importance in regulating various cell functions. They are synthesized from phosphatidylinositol by sequential phosphorylations on the several hydroxyls of the inositol ring to create polyphosphoinositides that function either as docking sites to promote formation of molecular signaling complexes, or serve as precursors for soluble inositol polyphosphates that act as diffusible intracellular messengers. Phosphoinositides are involved in the control of many processes, including membrane traffic, endo- and exocytosis, mitogenesis and apoptosis. Pharmacological tools have helped to clarify many details of phosphoinositide metabolism and have unveiled the roles of these lipids in the control of specific signaling pathways. However, because of their pleiotropic functions it has been questionable whether pharmacological manipulation of inositide formation and metabolism can be of therapeutic value. This review briefly summarizes the means by which inositide functions have been pharmacologically manipulated, and discusses possibilities for specifically targeting certain aspects of their regulatory functions.

I. INTRODUCTION

Myo-inositol was first identified in the middle of the 19th century as a component of muscle tissue. Later, it was found to be present in mycobacteria in a membrane lipid fraction that was subsequently identified as mannosides of phosphatidylinositol (PtdIns)[1] [1]. Phosphoinositides were first described in the ethanol-insoluble fraction of bovine brain by Folch, who also estimated that they contain inositol and phosphate in a ratio of 1:2 ("diphosphoinositide") [2]. PtdIns ("monophos-phoinositide") was subsequently isolated from other tissues including heart, liver and plants, but di- and triphosphoinositides were thought to be present only in brain until their detection later in all eukaryotic cells (see in [3]). The importance of *myo*-inositol as a dietary requirement was recognized in the 1950s, without any idea why animals and even cultured cells require its presence for normal development and growth (see [4] for review).

In 1953 Mabel and Lowell Hokin noticed that stimulation of the secretion of the exocrine pancreas was associated with a striking increase in the ^{32}P-phosphate labeling of a phospholipid fraction, that they later identified as phosphatidic acid and phosphatidylinositol [5]. This observation marked the beginning of an era which uncovered the role of inositol lipids in receptor-mediated Ca^{2+} signaling. A major breakthrough was when Robert Michell proposed that PtdIns breakdown by phospholipase C (PLC) enzyme(s) is an early signaling event that links activation of certain cell surface receptors to calcium-regulated intracellular responses [6]. The link between phosphoinositide breakdown and the generation of

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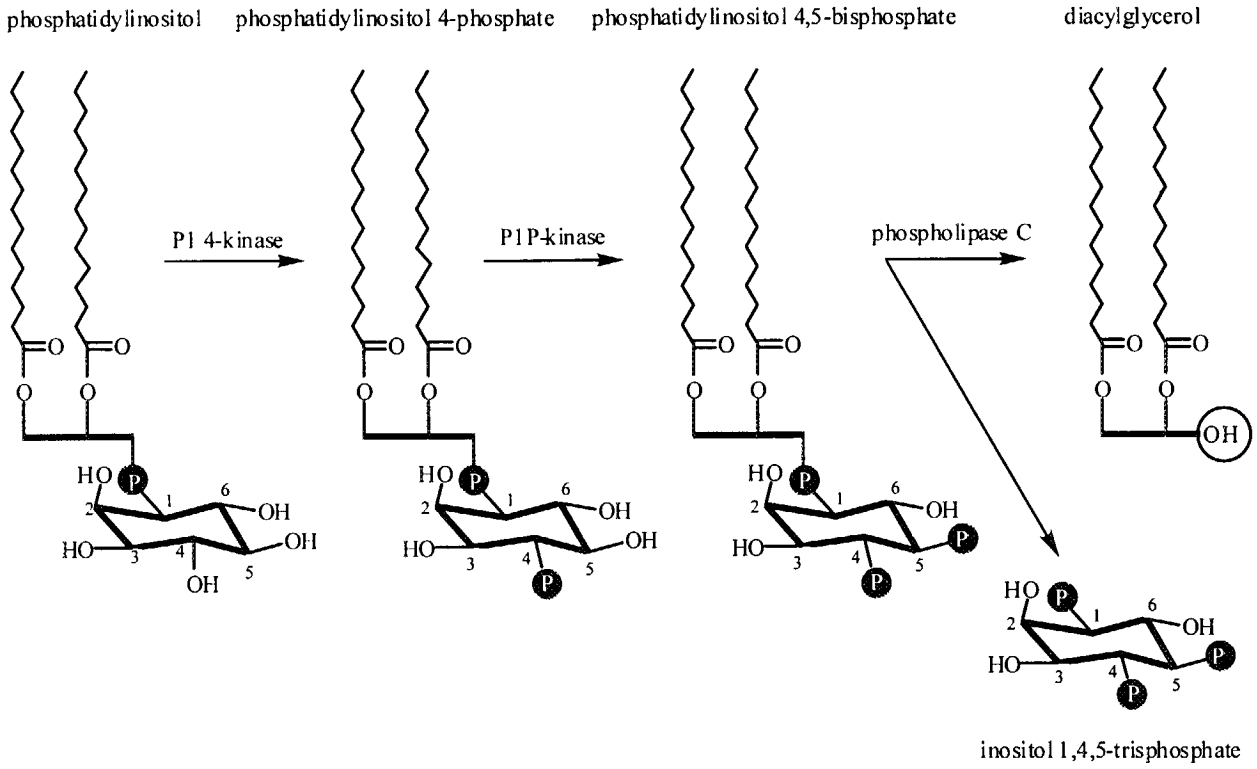


Fig. (1). The 'classical' phosphoinositides and the two second messenger products, diacylglycerol and inositol, 1,4,5-trisphosphate formed by the phospholipase C-mediated hbydrolysis of phosphatidylinositol 4,5-bisphosphate.

an intracellular Ca^{2+} signal was later found when PtdIns(4,5)P_2 was identified as the primary substrate of receptor-regulated phospholipase C (PLC) enzymes [7,8], and when the water soluble product of this reaction, Ins(1,4,5)P_3, (Fig. **1**) was found to release Ca^{2+} from intracellular stores [9].

Many details of this ubiquitous signaling system have been clarified in the last 20 years. Phosphoinositide-specific PLC enzymes, some regulated by G nucleotide-binding proteins and others by receptor tyrosine kinases, have been identified and cloned [10]. Impressive progress has been made in characterizing the protein kinase C enzymes that are the target of diacylglycerol (DAG), the other product of PtdIns(4,5)P_2 hydrolysis [11]. Another important area of research dealt with the metabolism of the water-soluble Ca^{2+} mobilizing messenger, Ins(1,4,5)P_3. In addition to its sequential dephosphorylation, this messenger compound undergoes 3-phosphorylation by a specific Ins(1,4,5)P_3 kinase, to yield Ins(1,3,4,5)P_4, a metabolite that serves as a messenger on its own right [12,13]. These studies, and the use of improved HPLC analysis for separation of stereoisomers of the water-soluble inositol phosphates, revealed the presence of several highly phosphorylated inositol compounds, such as InsP_5 and InsP_6, some even bearing pyrophosphate esters on their inositol ring, in eukaryotic cells [14-16]. The biological function(s) and the metabolic routes by which these highly phosphorylated inositols are formed are still an underexplored research area.

A new direction of phosphoinositide research began when a novel PI kinase[2] enzyme was described that phosphorylated PtdIns on the 3- rather than the 4-position of the inositol ring [17]. PI 3-kinases and their 3-phosphorylated lipid-products did not fit into the "classical" inositide-Ca^{2+} messenger scheme, but their regulation by receptor tyrosine kinases and their association with oncogenic tyrosine kinases fueled great interest to understand their biological functions. One important conclusion drawn from many studies was that 3-phosphorylated inositides are not precursors for inositol phosphates but rather

act in the membrane as signaling molecules [18]. This notion uncovered a new aspect of inositide function, namely the role of these lipids as membrane anchors to recruit, and regulate important signaling proteins. The functions of several proteins have been known to be influenced by inositides for some time, but only recently have some of the protein motifs that specifically interact with these lipids been identified [19].

Another important direction of inositide research investigates the function of phosphatidylinositol glycans, which anchor some proteins to the outer surface of the plasma membrane. PtdIns-glycan-anchored proteins are key determinants of the invasiveness of many pathogens including bacteria and protozoa, but are also important for mammalian cell functions. Differences between the synthetic machinery used by pathogens and mammalian cells have been targeted to develop agents that can fight infections. Because of its different scope, this review will not cover the physiology and pharmacology of inositide glycans, which can be found in recent reviews elsewhere [20].

This short summary already indicates the complexity of how inositides integrate in almost every aspect of cell regulation. This represents a major conceptual and experimental challenge: on the one hand, it is difficult to perceive how signaling specificity can be achieved at the cellular level with such a ubiquitously used mechanism. On the other hand, how can one target one aspect of inositide metabolism by a drug without affecting many other cellular processes. More information needs to be gathered before these questions can be answered. Nevertheless, the successful development of specific inhibitors of various forms of protein tyrosine kinases suggests that different classes of PI-kinases might be also targeted with specific inhibitors. Although most successful drugs interfere with the catalytic function of enzymes, there is at least a theoretical possibility (and a few examples) that drugs could be developed to prevent interactions between signaling proteins. This approach could provide a much greater degree of specificity and, therefore, will also be discussed at the end of this review.

The following is a brief overview of our current understanding of the various aspects of inositide research, each followed by a discussion of the pharmacological means by which they can be manipulated. Although all of these themes grew around the original "PI-cycle", each of them has now developed to a large field of its own. This review cannot cover all the details of these developments and, in each case, I will refer to recent reviews for more detailed information. Citation of original works are often omitted when only a summary of our understanding, based on the work of many contributing laboratories, is presented.

II. PHOSPOINOSITIDE SYNTHESIS

II.1. Synthesis of Phosphatidylinositol

Phosphatidylinositol is at the center of inositol lipid biosynthesis. It is the ultimate precursor of polyphosphoinositides after its sequential phosphorylation by various inositol lipid kinases, and also is the starting point for the synthesis of phosphatidylinositol glycans. As shown in Fig. **2**, PtdIns can also be hydrolyzed by PLC enzyme(s) (all forms of PLCs can use PtdIns as substrate *in vitro*), but it is not clear whether direct hydrolysis of PtdIns takes place in stimulated cells. PtdIns is synthesized from *myo*-inositol and CDP-diacylglycerol [21] by a reaction that is generally believed to take place in the endoplasmic reticulum (Fig. 2), based on early studies using conventional cell fractionation techniques [22]. Recently, PtdIns synthesis was found to take place also at the plasma membrane, as evidenced by cell fractionation studies and studies on turkey erythrocyte membranes [23,24]. The latter finding has important implications (see below) since it alleviates the need to transfer PtdIns from the ER to the other membrane(s) where its subsequent processing takes place.

Wherever PtdIns synthesis occurs, the two substrates of the PtdIns synthase enzyme needs to be provided. In mammalian cells, *myo*-inositol is either taken up from the extracellular medium, or is provided by the dephosphorylation of inositol phosphates. Lower organisms or plants can also

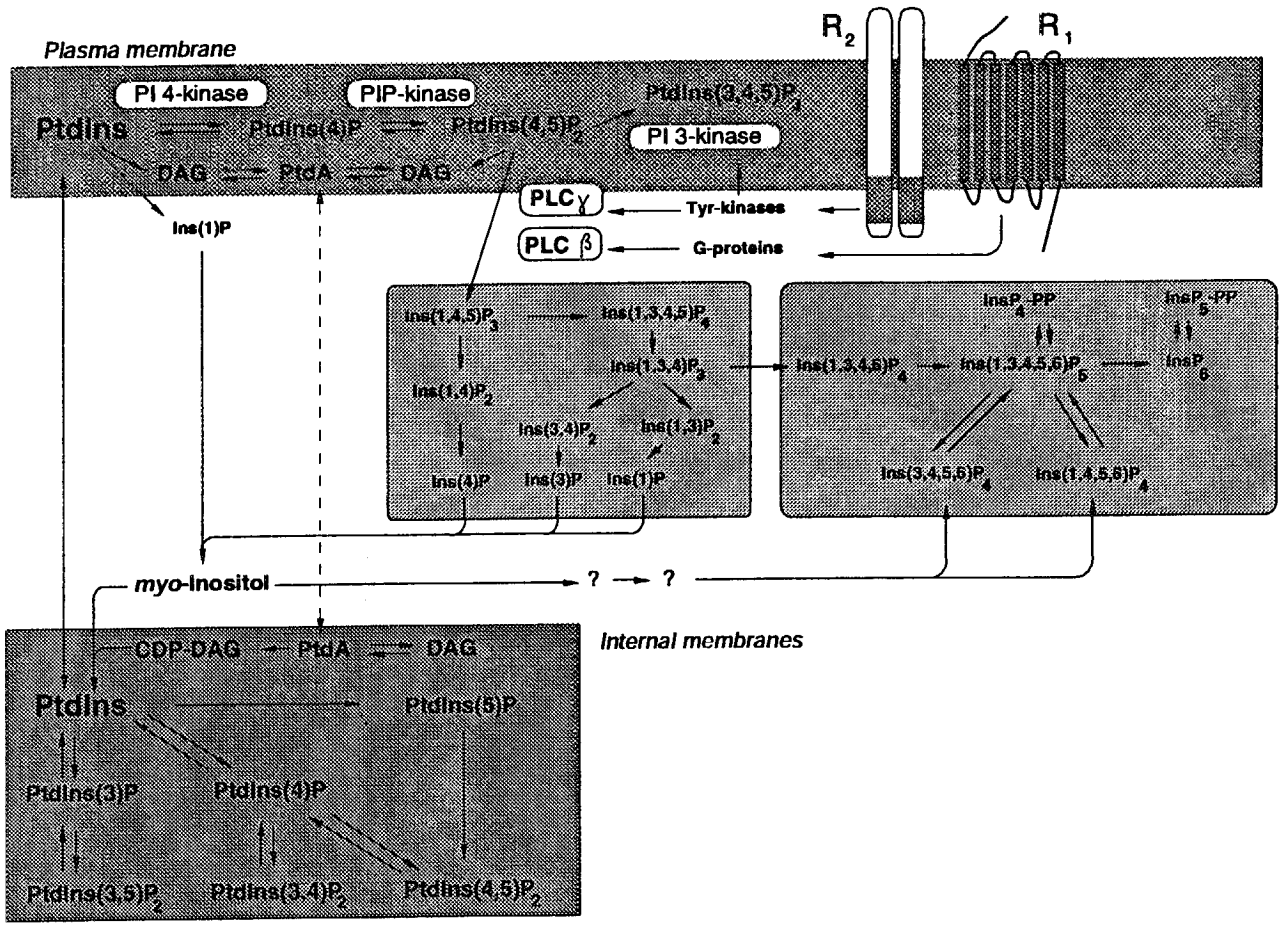


Fig. (2). Inositol lipid and inositol phosphate metabolism in mammalian cells. Phosphatidylinositol (PtdIns) is phosphorylated by various PI- and PIP-kinases to form numerous inositide isomers. Phosphatidylinositol 4,5-bisphosphate [PtfIns(4,5)P_2] is hydrolyzed by PLC enzymes activated by cell surface receptors to form inositol 1,4,5-trisphosphate [Ins(1,4,5)P_3] and diacylglycerol (DAG), both of which acts as second messengers. Ins(1,4,5)P_3 is rapidly metabolized by sequential dephosphorylations eventually yielding *myo*-inositol. DAG can also be recycled through phosphatydic acid (PtdA) to form CDP-DAG, which together with inositol serves as precursor for PtdIns resynthesis. See text for more details.

synthesize D-*myo*-inositol-3-phosphate (L-myo-inositol-1-phosphate) from glucose-6-phosphate [25]. It is noteworthy that *myo*-inositol does not cross the blood-brain barrier, so the brain almost entirely relies upon recycled *myo*-inositol [26]. The other substrate, CDP-DAG is formed from CTP and phosphatidic acid (PtdA), the latter originating either from *de novo* synthesized DAG, or from the DAG produced by phospholipid hydrolysis by PLC (Fig. **2**). Moreover, activation of PLD, which usually acts on phosphatidylcholine, yields PtdA directly. The DAG kinase that translocates to the plasma membrane in stimulated cells [27] rapidly converts DAG to PtdA, and this is why ^{32}P-labeling of PtdA was used as a relatively good measure of PLC activation in stimulated cells [6]. These are important details to keep in mind, since there are several examples where PtdIns synthesis is inhibited, in a secondary manner, due to a limited supply of substrates, and the direct target of the inhibitor is "upstream" in the cycle. While DAG formation is known to take place in the plasma membrane following stimulation, little is known whether DAG or PtdA can be formed in other membrane compartments. PLC enzymes as well as

their lipid substrates have been found in various intracellular membranes, and the role of PLD in Golgi function is well-documented [28]. On the other hand, the majority of CDP-DAG synthesis certainly takes place in the ER (and some in the mitochondria), but the question of whether it also occurs in other membranes remains to be answered. If not, then PtdA has to be transported between membranes for the proper organization of the various steps of PtdIns synthesis. These factors all contribute to the complexity of PtdIns synthesis, but also represent possible means by which substrate depletion and hence inhibition of PtdIns formation might be selectively achieved in certain membrane compartments (Fig. **2**).

It is quite remarkable that a PtdIns synthase enzyme has only recently been cloned based on complementation of a strain of Saccharomyces cerevisiae deficient in this enzyme activity [29]. Previously, a 21 kDa membrane-bound enzyme had been purified close to homogeneity [30], but no peptide sequences could be obtained from these efforts, and it is quite possible that multiple enzymes can perform this reaction.

II. 2. Synthesis of Polyphosphoinositides

Once PtdIns is synthesized it can be phosphorylated by PI- and PIP kinases at the various positions of the inositol ring. Classically, the synthesis of PtdIns(4,5)P_2, the major PLC substrate was believed to take place by sequential 4- and 5-phosphorylations of PtdIns and PtdIns(4)P by separate PI 4-kinase(s) and PtdIns(4)P 5-kinase(s) in the plasma membrane. However, in the last ten years, several PI kinases have been isolated and cloned, and the variety of inositol lipids and the number of membrane-linked cellular processes that they regulate have grown beyond any expectation.

PI 4-Kinases

The enzymes that phosphorylate PtdIns on the 4-position of the inositol ring, called PI 4-kinases, have been classified into two major groups based on their catalytic properties: The **type II enzymes** are tightly membrane-bound proteins, with high affinity for ATP (K_m: in the10-50 μM range) and hence high sensitivity to inhibition by adenosine (K_i: in the 10-70 μM range). Their PI 4-kinase activity is greatly stimulated by detergents, and requires Mg^{2+} (or Mn^{2+}) but is inhibited by μM concentrations of Ca^{2+}. These enzymes have been purified from various membrane sources yielding a protein of about 56 kDa that can be renatured from SDS gel slices. The identification and molecular cloning of the type II PI 4-kinase(s) is still to be accomplished. **Type III PI 4-kinases**, on the other hand, are larger proteins with low affinity for ATP (K_m: in the 400-800 μM range), low sensitivity to adenosine (K_i: above mM), and with only loose attachment (for most part) to the membranes [31]. Two such enzymes have been cloned, a smaller (~100 kDa) form that is termed PI 4-kinase β, and a larger (~200 kDa) species, which appears to also have a smaller (92 kDa) splice variant, termed PI 4-kinase α. Homologues of the two type III mammalian PI 4-kinases are present in yeast as well as in plants. Their separation so early in evolution, together with their remarkable conservation, indicates that they serve distinct and fundamentally important functions [32]. Recent reviews provide a more detailed summary of our current knowledge of these proteins [33-35].

PI 3-Kinases

A whole family of PI kinases (originally termed type I PI kinases) were found to phosphorylate PtdIns on the 3- rather than the 4-position of the inositol ring [17]. The catalytic properties of the PI 3-kinases show many similarities to those of type III PI 4-kinases, although their K_m for ATP (100 μM range) is lower than that of the type III enzymes. They are also less sensitive to inhibition by adenosine than type II PI 4-kinases. Based on the substrates that they can phosphorylate *in vitro*, PI 3-kinases are classified into three groups. **Class I** enzymes are able to utilize either PtdIns, PtdIns (4)P or PtdIns (4,5)P_2 as substrates *in vitro* to produce the corresponding 3-phosphorylated lipids, but their primary substrate within the cells appears to be PtdIns (4,5)P_2. This is the group that have been studied the most, and whose regulation is best understood. Three members of this class (also called group 1A), PI3Kα, PI3Kβ

and PI3Kδ are ~ 110 kDa proteins with significant sequence similarities to PI4Kβ within their catalytic domain. Their activity and cellular localization is regulated by a closely associated p85 regulatory subunit, which posesses two SH2 and one SH3 domains for interaction with tyrosine-phosphorylated and proline-rich sequences, respectively. Upon activation by growth factors or soluble tyrosine kinases, p85 brings the catalytic subunit to cellular membranes where p85 itself undergoes tyrosine phosphorylation leading to an increased catalytic activity of the p85/p110 complex. Smaller splice variants of p85 lacking the SH3 domain, named p55 and p50, have also been described, but less is known about their specific regulatory features. A fourth and somewhat different member of the Class I PI 3-kinase family (termed group 1B) is PI3Kγ, which has sequence similarities to the other members but is primarily regulated by βγ subunits of heterotrimeric G proteins, either directly or via another adapter protein, p101 [36,37]. An interesting and distinctive feature of PI3Kγ is that it is able to regulate the activity of the MEK/MAP-kinase cascade via its protein kinase activity [38]. While all PI 3- and 4-kinases have been shown to autophosphorylate and in many (although not all) cases autophosphorylation was found to inhibit the enzymes's lipid kinase activity, PI3Kγ is the only member whose protein kinase activity has been linked to a regulatory process. All four members of the Class I enzymes can be regulated by Ras via a Ras binding domain that is found within the N-terminal half of their sequences. **Class II** PI 3-kinases can only use PtdIns or PtdIns (4)P, but not PtdIns (4,5)P_2 as substrate. These enzymes are larger (170-220 kDa) proteins, which contain the characteristic lipid kinase catalytic and lipid kinase unique domains, but also possess a C2 domain in their C-terminus that could mediate Ca^{2+} and phospholipid regulation of the enzyme. Little is known about the regulation of the Class II PI 3-kinases and about the processes in which they play a regulatory role. **Class III** PI 3-kinases only phosphorylate PtdIns *in vitro* and this substrate restriction also has been demonstrated *in vivo*. The first such enzyme was described in *Saccharomyces Cerevisiae* as the product of the VPS34 gene, and its function is crucial in vacuolar sorting [39]. The membrane recruitment of Vps34p is mediated by association with the VPS15 gene product, a serine/threonine kinase that also activates the lipid kinase activity of Vps34p. Mammalian homologues of Vps34p and Vps15p, termed PtdIns 3-kinase and p150, respectively, have also been described, but similar to the Class II kinases, relatively little is known about their function(s) and regulation in mammalian cells.

Knockout studies on PI 3-kinases indicate that elimination of the 110 kDa catalytic subunit of PI 3-kinase α is an embryonic lethal [40]. The total elimination of the p85α adaptor protein, together with its smaller splice variants, also results is neonatal death [41]. Elimination of p85 α (but not of its smaller splice variants, p55) causes severe B-cell immunodeficiency [42] and altered insulin responsiveness [43]. In contrast, p85β knockouts do not present with a specific phenotype [44]. Complete disruption of PI3Kγ is associated with greatly impaired neutrophil and macrophage, but not B cell, functions [45-47], and an increased incidence of colorectal cancer [48]. These results suggest that different PI 3-kinases may have evolved to serve distinct biological functions, and that their selective targeting by pharmacological means would be quite a meaningful goal. More details on PI 3-kinases can be found in recent reviews [44,49].

PI kinase related kinases are a group of serine/threonine kinases that show significant sequence homology with PI 3-kinases and type III PI 4-kinases [50]. These enzymes are large proteins, and are mostly involved in cell-cycle control, related to DNA damage and repair. The most prominent member of this group is the ATM protein, the mutation of which is responsible for the human disease, ataxia teleangiectasia [51]. Additional kinases, like the DNA-dependent protein kinase [52], and the rapamycin target proteins (TORs and its mammalian forms, mTORs or FRAP) belong to this family [53]. Since they do not seem to phosphorylate inositol lipids, they are discussed in this review only because of their sensitivity to higher concentrations of the PI 3-kinase inhibitors, wortmannin and LY294002 (see below).

PIP Kinases

Originally, all PIP kinases were believed to phosphorylate PtdIns(4)P to PtdIns (4,5)P_2. However, other isomers of PtdInsP, namely, PtdIns(3)P and more recently, PtdIns(5)P, have been found in cells and this, together with the ambiguity concerning the true physiological PtdInsP substrate(s) of the enzymes, required adjustments about their nomenclature (see [54] for details). Isolation of PIP kinase activities from red blood cells revealed two type of enzymatic activities, called type I and and type II PIP kinases. The **type I** enzyme was of larger size (68 kDa) than the **type II** (53 kDa) [55]. When another PIP kinase, immunologically related to type I, but of significantly larger size (90 kDa), was isolated from rat brain, the latter protein was named Ib, while the smaller, 68 kDa, form was designated as Ia. Catalytically, the main distinctive feature of the type I enzymes was their selective stimulation by phosphatidic acid *in vitro* as opposed to the type II form which did not show this regulation [56].

Molecular cloning of type I and type II enzymes revealed an even greater multiplicity, and α, β and γ forms of both the type I and the type II enzymes have been isolated (see[57] for details). All three forms of type II (α, β and γ) are about the same molecular size and correspond to the 53 kDa activity originally described. The type Iα and type Iβ forms probably correspond to the smaller, 68 kDa Ia enzyme, while type Iγ may represent the larger, Ib PIP kinase. Homologues of these enzymes have been identified in yeast, encoded by the genes MSS4 and FAB1. Mssp4p is most likely to be the homologue of the mammalian type I PIP kinase, but Fab1p has a unique substrate specificity and phosphorylates only PtdIns(3)P to form PtdIns(3,5)P_2. This lipid has only been recently identified in mammalian cells and its level was found to change after osmotic challenge [58]. The mammalian form of Fab1p has also been isolated, as a 235 kDa protein (p253) which shows extensive sequence homology with the yeast enzyme [59]. Because of their unique substrate specificity, this group of enzymes was proposed to be termed type III PIP kinases [57,60].

Both the type I and type II PIP kinases are believed to synthesize PtdIns(4,5)P_2, but only recently has it become clear that they act upon different substrates, PtdIns(4)P and PtdIns(5)P, respectively [61]. Given the very low level of PtdIns(5)P observed in mammalian cells, it is not clear what function(s) the quite abundant type II PIP kinases can have, and what significance it has to produce PtdIns(4,5)P_2 in two alternative pathways. It is important to note that the substrate specificity of the various PIP kinases can be significantly different *in vitro* or *in vivo*. This has been elegantly demonstrated in a recent study where a yeast complementation assay was used to determine the "true" substrate profile of the type I and type III PIP kinases. The type I enzyme had been found to use PtdIns, PtdIns(3)P [besides PtdIns(4)P] to produce PtdIns(5)P, PtdIns(3,4)P_2 and PtdIns(3,5)P_2 in addition to PtdIns(4,5)P_2 *in vitro* [62]. However, these studies revealed that the enzyme's *in vivo* function is to convert PtdIns(4)P to PtdIns(4,5)P_2 [60]. Similar studies are needed in mammalian cells to determine the real substrate profile of these enzymes. More details about PIP kinases are available in recent reviews [54,57].

II. 3. Inhibitors of Phosphoinositide Synthesis

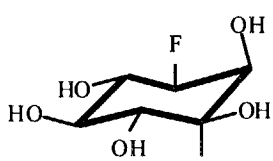
Inhibitors of PtdIns Synthesis

Limiting substrate availability and inhibition of the PtdIns synthase enzyme are the two obvious ways to achieve inhibition of PtdIns synthesis. To compromise the supply of *myo*-inositol one could inhibit its recycling from inositol phosphates by inhibition of the inositol phosphate phosphatases. This is the mechanism by which lithium inhibits PtdIns synthesis (see below), but this effect is indirect and will be discussed in more detail under inhibitors of the inositol phosphatases. Since *myo*-inositol is taken up by cells by a *myo*-inositol/Na^+ co-transporter, it is possible to inhibit *myo*-inositol uptake into cells and it has been shown that monosacharides, such as glucose, L-fucose [63], as well as 3-substituted analogs of *myo*-inositol [22] can act as *myo*-inositol uptake inhibitors. While these compounds have been used

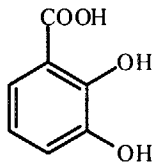
in cell culture studies with some success, they cannot be effectively used at the prevailing *myo*-inositol concentrations of the extracellular fluid, and did not gain enough promise to be pursued as an inhibitory strategy. Similarly, *myo*-inositol analogs, including chlorinated cyclohexanes [64] or *myo*-inositols substituted at various positions of their OH groups [65] have been tested as substrates or inhibitors of PtdIns synthesis. Of these analogs, only the 3- and 5-OH substituted compounds are taken up by cells and incorporated into PtdIns, although with lower efficiency than *myo*-inositol [65,66]. Interestingly, high affinity *myo*-inositol uptake has been found to decrease due to diminished expression of the transporter in astrocyte-like cells after treatment with either lithium, carbamazepine or valproate, and it was speculated that this could be a common mechanism of their effectiveness in the treatment of manic-depressive disease [67]. Because of the importance of 3-phosphorylated inositides in the regulation of cell growth and proliferation, theoretically, 3-substuted *myo*-inositol analogs can be utilized to antagonize PtdIns $(3,4,5)P_3$-mediated cellular responses. Indeed, 3-substituted derivatives (Fig. **3**, compound **1**) **(3-deoxy-3-fluoro-, 3-deoxy-3-chloro-, and 3-deoxy-3-azido-*myo*-inositol)** have been shown to act as antimetabolites of *myo*-inositol and were found to inhibit the growth of *v-sis*-transformed but not normal NIH 3T3 cells [68,69]. The reported IC_{50} values of these compounds are in the 40-150 μM range, and their effects can be antagonized by *myo*-inositol, which limits their efficient use.

Inhibitors of PI- and PIP Kinases

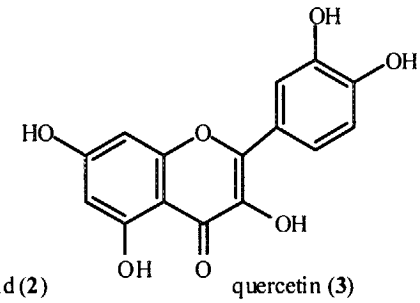
The old rule that kinases are the best targets of inhibitors also holds for the field of inositides, even though no good inhibitors of PI kinases were identified for a long period of time. As discussed above, **adenosine** and **5'-chloro-5'-deoxyadenosine** were found to potently inhibit type II PI 4-kinases, but these substances penetrate poorly into cells and were used only as *in vitro* tools to classify and inhibit the enzyme(s). A search for microbial metabolites that inhibit a PI 4-kinase of A431 cell membranes (presumably the type II PI 4-kinase) identified **2,3-dihydroxybenzoic acid** (**2**) as a relatively potent inhibitor of the enzyme (Fig. **3**). Other analogs were less effective, but 2,3-dihydroxybenzaldehyde was slightly more potent [70]. Other inhibitors of PI-kinases were found among isoflavones (such as **orobol**, but not genistein) and flavones (such as **quercetin** (**3**) and fisetin) [70], all competing for the ATP-binding site of the enzyme. Quercetin was also found to inhibit PI 3-kinases (IC_{50} 3.8 μM) [71], and its analogs as well as other chromones have been tested for PI 3-kinase inhibition. This research effort has led to the identification of the first rationally selected PI 3-kinase inhibitor, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (**LY294002**) (IC_{50}: 1.4 μM) [72] (Fig. **4**, compound **4**). LY294002 completely and reversibly inhibits PI 3-kinase(s), but can also inhibit the structurally related type-III (but not type II) PI 4-kinases at higher concentrations (IC_{50}:100 μM) [73]. It does not inhibit several protein kinases, such as PKA, PKC, MAP-kinase, EGF-receptor, c-src tyrosine kinase [72]. LY294002 rapidly became one of the most widely used PI 3-kinase inhibitors. However, recently LY294002 was found to inhibit casein kinase II as potently as it inhibits PI 3-kinase, so can also have additional targets [74].



D-3-deoxy-3-fluoro-*myo*-inositol (**1**)



2,3-dihydroxybenzoic acid (**2**)



quercetin (**3**)

Fig. (3).

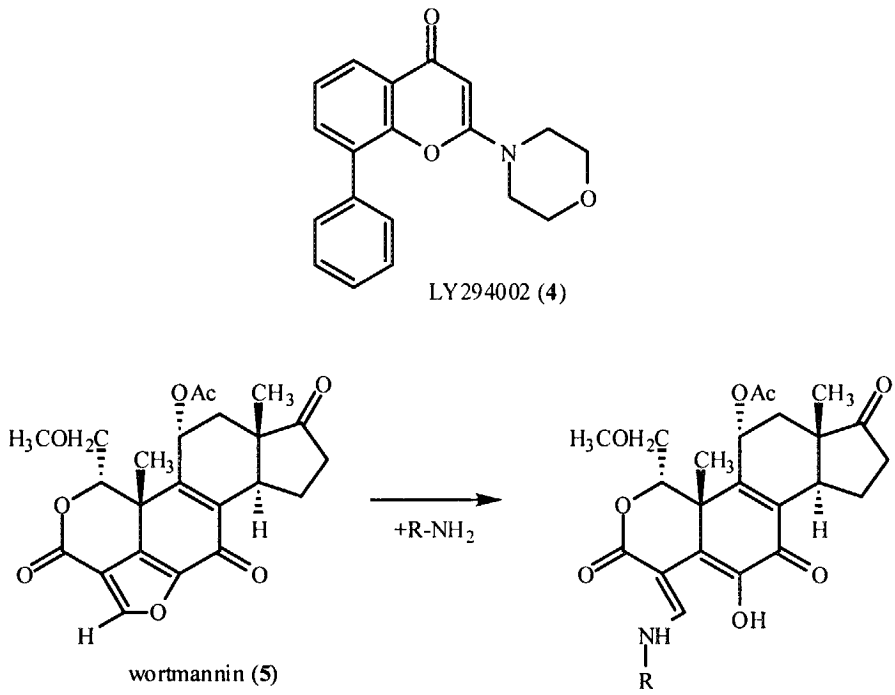


Fig. (4).

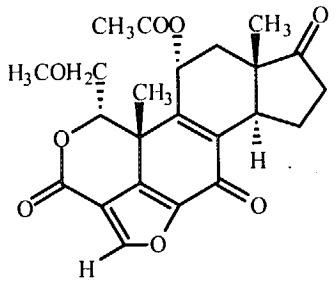
The other PI 3-kinase inhibitor, **wortmannin** (Fig. **4**, compound **5**), had been known for a period of time before its site of action was revealed. It was first described as an antiinflammatory agent isolated from *Penicillium* [75] that strongly inhibited neutrophil cell function(s) [76]. Most notably it was found that wortmannin (or its 17-hydroxy analog) is an extremely potent inhibitor (IC_{50}: 1-10 nM) of the respiratory burst in neutrophils, macrophages and monocytes [77], but its target remained elusive. Wortmannin was rediscovered in 1992 as a potent myosin light chain kinase inhibitor isolated from cultures of the fungus *Talaromyces wortmannii* [78]. Comparisons of the inhibitory concentrations of wortmannin on myosin light chain kinase and on secretion in chromaffin cells, RBL-2H3 cells, and human basophils, showed that inhibition of secretion in the latter two cell types occurs with nM concentrations of the drug, which is several magnitudes lower than those required to inhibit myosin light chain kinase or secretion from chromaffin cells (μM) [79,80]. Studies from three independent laboratories almost at the same time came to the conclusion that wortmannin inhibits PI 3-kinase. This is the main mechanism by which the compound exerts its effects at low, nanomolar concentrations in those secretory immune cells [80-82].

It has been noted from early studies that the potency of wortmannin depends on the incubation time with the inhibitor before a response is initiated. This phenomenon is due to the fact that wortmannin interacts covalently with selected amino- and thiol groups of its target proteins after the nucleophilic opening of its furan ring [83] (Fig. **4**). In the case of the p110 catalytic subunit of PI 3-kinase α, wortmannin covalently binds to Lys-802 [84], a residue that is highly conserved within the catalytic domains of PI 3-kinases and their related kinases [35]. The similarity between the catalytic domains of PI 3-kinases, the type-III PI 4-kinases, and the PI kinase-related kinases (see above) explains why the latter group of enzymes are also inhibited by wortmannin, although only at higher (μM) concentrations. Even PI 3-kinases seem to differ in their wortmannin sensitivities: for example, Vps34p, the yeast homologue of Class III PI kinase, has a significantly lower sensitivity (IC_{50}: 3 μM) [85] than Class I PI 3-kinases (IC_{50}: 1-6 nM). Species differences also exist: the two mammalian type III PI4-kinases have similar affinities to wortmannin (IC_{50}: 100 nM), but of their two yeast homologues, only Stt4p but not Pik1p is sensitive to wortmannin in the same concentration range [86]. These differences indicate that the proper positioning of wortmannin within the catalytic domain for the nucleophilic

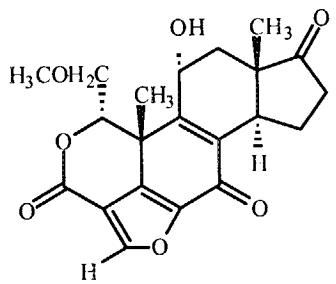
attack of its furan ring is a major determinant of its reactivity, even if we assume that the analogous conserved Lys-residue serves as a target for covalent modification in each of the enzymes. Most recently the crystal structure of P13Kγ complexed with various inhibitors, including wortmannin, became available [87]. This study provides invaluable information for the rational design of newer inhibitory compounds and together with similar structural information on other PI kinases (or PI-kinase related kinases) it will help to determine whether it is feasible to design more specific inhibitors of the various classes of PI kinases.

Only a few attempts have so far been reported in which the effects of various wortmannin analogs (Fig. **5**) were compared on PI 3-kinases. Two analogs of wortmannin, 11-O-deacetylwortmannin (WM-4)(**6**) and 16-methylidenwortmannin (WM-25) (**7**) were about ten-fold less effective than wortmannin in inhibiting PI 3-kinase, while derivatives in which the furanoid ring is open displayed very low potency [80]. Another wortmannin derivative, KT7692 (**8**), had significantly lower potency against PI 3-kinase than wortmannin, with an unchanged potency toward myosin light chain kinase [88]. We compared some of these inhibitors on the two mammalian type-III PI 4-kinases (α and β) and found that their relative potencies were different for the two enzymes and differed even more from their relative potencies reported on PI 3-kinases (S. Kim, and T. Balla, unpublished observations). This suggests that it is feasible to develop inhibitors that would discriminate between the various classes of PI 3- and 4-kinases. In another study, substitutions have been made on the 11-O-acetyl group of wortmannin, which (as in **9**) improved its inhibitory potency against PI 3-kinase (3-4-fold decrease in IC_{50}) with enhanced cytotoxicity [89]. Unfortunately, no data are available with these inhibitors for other PI 3- or type-III PI 4-kinases (or the PI-kinase-related kinases) to assess their relative specificities.

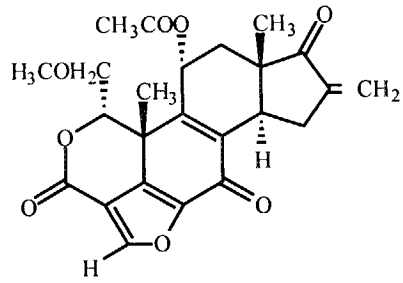
A major difficulty in comparing the potencies of wortmannin and its derivatives on various kinases and on different cellular responses is the reactivity of the compound with other proteins



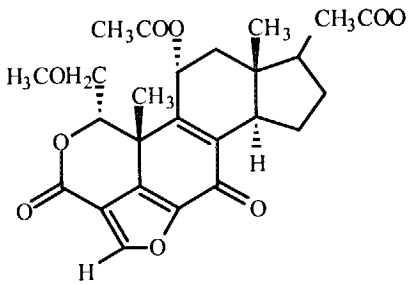
wortmannin (**5**)



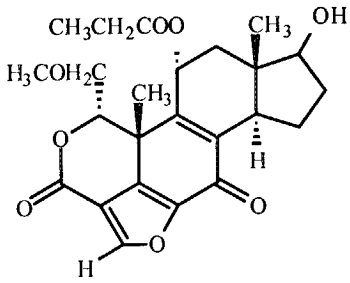
11-O-desacetoxylwortmannin (WM-4) (**6**)



16-methylidenwortmannin (WM-25) (**7**)



17-dihydro-17-O-acetylwortmannin (KT7692) (**8**)



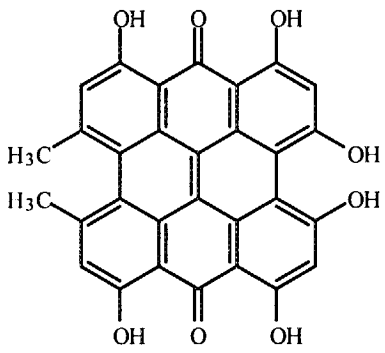
11-O-desacetyl-11-O-propionyl-17-dihydrowortmannin (**9**)

Fig. (5).

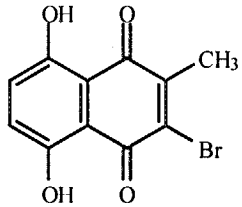
and its labile nature in aqueous solutions. The true concentration of wortmannin at a cellular target can be significantly lower than calculated if the protein concentration is high (such as in concentrated cell suspensions), or if the aqueous dilution is not made immediately prior to the addition of the compound to the cells or enzymes. Moreover, because of the covalent binding to its targets, even lower concentrations of wortmannin can affect alternative protein targets in prolonged incubations. Likewise, wortmannin might have been reacted with other proteins by the time a slower cellular response develops. In addition, the potency of wortmannin can be affected by the ATP concentration, relative to the K_m of the enzyme of interest, which will also affect the sensitivity of an enzyme to the compound. All these factors should be kept in mind when concluding about the correlation (or lack of it) between wortmannin-sensitivities of enzymes and of cellular responses.

In summary, wortmannin and LY294002 have already proven to be invaluable tools to analyze the role of inositol lipid kinases in cell regulation. They should serve as templates for the development of newer compounds that would better discriminate between the various groups and classes of PI kinase enzymes. Structural information on the various forms of PI kinases would greatly aid such an effort.

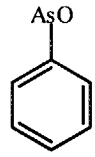
Additional inhibitors of PI 3-kinases, such as **hypericin** (Fig. **6**, compound **10**) (IC_{50}: 0.2 μM) and uttronin (IC_{50}: 1 μM) have been identified among natural products [90]. Hypericin, (which is present in the extract of St John's wort) is also cytotoxic, especially after photoactivation but it is not known whether its biological effects are related to PI 3-kinase inhibition since it also inhibits EGF receptor tyrosine kinase [91]. **Halogenated naphtoquinones** (**11**) related to methyljuglone were also found to inhibit PI 3-kinases [92]. However, little is known about the specificity and usefulness of these compounds in cellular studies. Phenylarsine oxide (**PAO**) (**12**), which inhibits several proteins by reacting with their SH_2 groups, and **quercetin** (**3**) were both found to inhibit the PI 4-kinase that is associated with secretory granules of adrenal chromaffin cells [93]. Both inhibitors were able to prevent the resynthesis of PtdIns(4,5)P_2 in intact cells after Ca^{2+}-induced, PLC-mediated hydrolysis of the lipid [94]. Based on measurements in permeabilized adrenal glomerulosa and NIH 3T3 cells, our experience is that PIP 5-kinase(s) is even more sensitive to inhibition by quercetin than the PI 4-kinases; 10 μM quercetin already inhibits PtdIns(4,5)P_2 synthesis without affecting PtdInsP synthesis. In contrast, neither inhibitor was found to significantly inhibit the wortmannin-insenitive PI 4-kinase activity solubilized from adrenal membranes [presumably type II PI 4-kinase(s)] (P. Varnai and T. Balla unpublished observations). Altogether, these latter compounds are not specific enough to be used as inhibitors of PI kinases [74]. However, they could still be useful to discriminate between various classes of PI-kinase enzymes, and may help to identify or rule out participation of a particular PI kinase in a certain signaling pathway. More experiments are needed to determine the



hypericin (**10**)



halogenated naphtoquinone (**11**)



phenylarsine oxide (**12**)

Fig. (6).

sensitivity profile of these inhibitors on the various inositide kinases.

III. HYDROLYSIS OF PHOSPHOINOSITIDES

III. 1. Phosphoinositide-specific PLC

As mentioned before, the major regulated step in phosphoinositide metabolism is the phospholipase C-mediated hydrolysis of phosphoinositides to diacylglycerol and the respective inositol phosphates. Several forms of phosphoinositide-specific PLC enzymes have been purified, and initial attempts used the membranes (which was a logical choice since the enzyme works on its membrane-bound substrates) as a source to isolate the activity. However, ironically, all of the cloned PLC-s were purified from soluble fractions of brain homogenates [95], eventually leading to the cloning of three major forms of the enzyme, PLCβ, -γ and –δ[10]. Although these enzymes show a large degree of similarity and conservation within their X and Y catalytic domains, they markedly differ in their regulatory properties.

PLCβ enzymes (β_{1-4}) are the forms that are regulated by heterotrimeric G proteins and respond to activation of serpentine receptors. Two of these isoforms (β_1,β_3 but less often β_2) are activated by the α-subunits of the $G_{q/11}$ family of heterotrimeric G proteins,\ and β_2 is primarily activated by βγ subunits. Cells of hematopoetic origin contain relatively high amounts of the β_2 enzyme that is stimulated by the βγ subunits liberated from abundant G_i/G_o proteins, and this explains why pertussis toxin treatment almost completely eliminates PLC activation via their G-protein coupled receptors in such cells. In contrast, in most cells, G protein-coupled receptors activate PLC in a pertussis toxin-insensitive manner, via the $G_{q/11}$ proteins. The PLC-β forms are also found in association with cellular membranes, and the presence of at least some of the β_1 enzyme in the nucleus raised the possibility that this is the major form that is involved in nuclear inositide metabolism [96]. PLCβ contains a much longer C-terminus than the other isoforms and this part of the sequence is responsible for its regulation by G proteins. The PLC of *Drosophila melanogaster* (NorpA) plays a crucial role in invertebrate visual signal transduction [97] and is closest to the β-form of the mammalian PLC-s.

$PLC\beta_1$ knockout mice die early from epileptic-type seizures [98]. These are probably due to impaired signaling from M1 and M3 muscarinic receptors, which affects the balance between the stimulatory and inhibitory pathways in the brain. $PLC\beta_4$ knockout also results in neurological symptoms that suggest cerebellar dysfunctions [98]. In contrast, $PLC\beta_2$-deficient mice have no major abnormalities, although chemokine signaling is impaired in their neutrophil cells [99]. These cells as well as those originating from $PLC\beta_2/\beta_3$ knockout mice [47], still show normal or enhanced chemotactic responses, suggesting that this response does not require $Ins(1,4,5)P_3$ and Ca^{2+} increases. Elimination of $PLC\beta_3$ was reported to be an embryonic lethal [100] at an even at earlier stage (E2.5 days) than observed in $PLC\gamma_1$ knockout mice (E9 days) [101]. However, in another study $PLC\beta_3$ deficient mice developed normally and developed multifocal skin ulcers after 6 month of age [47].

PLCγ enzymes (γ_1 and γ_2) are activated by receptor tyrosine kinases. These enzymes contain an extra stretch of regulatory sequence inserted in between the two parts of their conserved catalytic domains (X and Y). This insertion contains two SH2 and one SH3 domains sandwiched in between the two halves of what appears to be a pleckstrin homology (PH) domain. PLCγ binds to phosphorylated tyrosines of several growth factor receptors or adapter molecules, thereby getting recruited to the plasma membrane where it undergoes phosphorylations on crytical tyrosine residues [102]. Tyrosine phosphorylation and membrane association increases the activity of the enzyme, probably through association with other regulatory proteins. PLCγ has also been shown to be regulated by tau proteins, in the presence of which its activity can be greatly enhanced by arachidonic acid [103]. Whether this regulation occurs under physiological conditions is yet to be determined. An additional feature of PLCγ is its

regulation by PtdIns(3,4,5)P_3, a feature that is especially prominent in PLCγ_2 activation in immune cells [104]. This is believed to be the result of increased recruitment of PLCγ to the membrane via interaction of its PH domain with PtdIns(3,4,5)P_3 of the plasma membrane [105]. (All of the PLC isoforms contain a PH domain close to their N-terminus, and this PH domain of PLCγ is in addition to the half one mentioned above). In other studies, the SH2 domains of PLCγ were implicated in PtdIns(3,4,5)P_3-mediated activation [106]. The complexity of PLCγ regulation is also indicated by evidence showing that the actin-binding protein, profilin, differentially regulates the tyrosine-phosphorylated and unphosphorylated forms of the enzyme [107]. Moreover, PLCγ phosphorylation and activation can also be observed after stimulation of GPCR-s, which probably occurs via transactivation of growth factor receptors. As mentioned above, PLCγ_1 knockout in mice is an embryonic lethal [101], but the embryonic fibroblasts show growth factor responses and can proliferate.

PLCδ (δ_1-$_4$) enzymes are the smallest members of the cloned PLC-s, and the least is known about their regulation. The crystal structure of this enzyme has been resolved (less the PH domain, whose structure was solved separately [108]) which helped to understand the molecular architecture of all PLC-s [109]. This enzyme (like all the other PLC-s) is regulated by Ca^{2+} ions in the concentration range that occurs during stimulation of cells, and it is argued that this is the isoform of PLC that is primarily regulated by cytosolic Ca^{2+} increases. The enzyme binds to membrane PtdIns(4,5)P_2 via its N-terminal PH domain, and this lipid also is the primary substrate of the enzyme. Since the PH domain of PLCδ_1 also binds Ins(1,4,5)P_3 with high affinity, elevated Ins(1,4,5)P_3 levels inhibit the enzyme's activity via interference with its localization to the lipid membrane [110]. Lower eukaryotes, such as yeast contain only one PLC gene (PLC1) and this enzyme is most homologous to PLCδ [111]. Recently an Ins(1,4,5)P_3-binding protein, termed p130, has been isolated and cloned and has great sequence similarity to PLCδ_1 and to the yeast Plc1p, without PLC catalytic activity. This is due to natural substitutions in key residues within the catalytic domain of p130. The biological function of this protein is unknown, but due to its high affinity to Ins(1,4,5)P_3 it was speculated that it may serve as a buffer to dampen the changes in Ins(1,4,5)P_3 levels [112]. More details on PLC isoforms and their regulation can be found in a very comprehensive recent review [113].

III. 2. Inhibition of PLC

Although PLC activation is one of the earliest steps in the signal transduction cascade in response to several stimuli, there are no good inhibitors of the enzyme available as yet. This is in spite of the fact that inhibition of PLCγ_1 could be an effective way of inhibiting cell proliferation and tumor growth and, therefore, has been used to screen for identifying natural product-inhibitors of the enzyme that would also inhibit tumor growth [114]. Aminoglycosides, such as **neomycin** (a mixture of Neomycin A, B and C) (Fig. **7**, compound **13**) and **gentamicin** have been shown to inhibit PLC activation [115,116], but these compounds do not act on the enzyme itself. Most likely, their effect is due to their binding to inositol lipids at the membrane [117,118], thereby obstructing substrate access to the PLC enzymes. While neomycin certainly may act via binding to PtdIns(4,5)P_2, long incubation times with high concentrations of the compound are required to reach high enough concentrations within intact cells [119]. On the other hand, aminoglycosides also interfere with Ca^{2+} binding to the outer surface of membranes by taking the place of Ca^{2+} in Ca^{2+}-binding proteins. This is quite prominent in the case of the Ca^{2+}-sensing GPCR, where neomycin is used as a Ca^{2+} agonist (e.g. [120]). Similar effects of neomycin could affect the function of certain Ca^{2+} channels and this should be kept in mind when using this inhibitor to alter PLC function since Ca^{2+} plays an important role in regulating the activity of all PLC enzymes.

The most widely used inhibitor of PLC activation is the aminosteroid, **U-73122 (14)** (Fig. **7**). This compound was found during a search for PLA_2 inhibitors at Upjohn, and was characterized

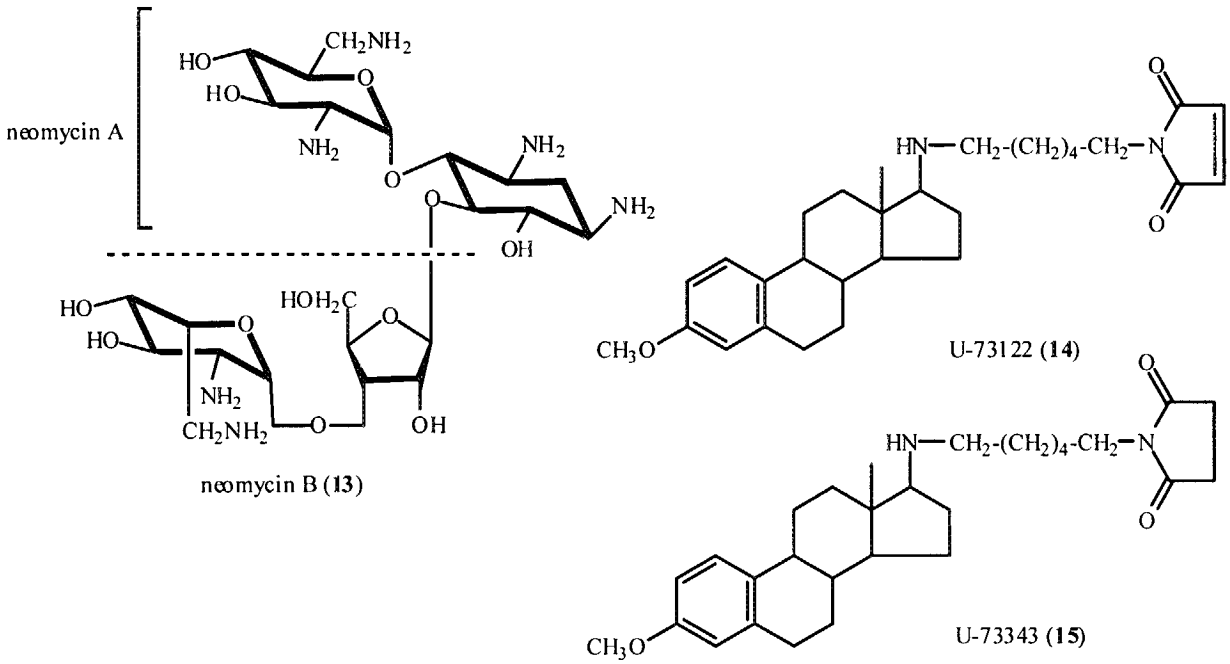


Fig. (7).

as a compound that inhibited PLC by a mechanism that, at that time, was best explained by interference with the Ca^{2+} binding of PLC in relation to substrate presentation to the enzyme [121]. Remarkably, the inhibitory activity of U-73122 shows extremely stringent structural requirements, as a highly similar structural analogue, lacking a double bond in the pyrrol ring, (U-73343) (**15**), was found to be completely ineffective. Unfortunately, no follow up studies have been published on the mechanism of action of U-73122, and instead, the compound has become known as a "PLC-inhibitor". U-73343 serves as its inactive, negative control and it also has been widely used as such. Indeed, U-73122 (but not U73343) has been shown to inhibit receptor-mediated formation of IP_3 and DAG and the subsequent Ca^{2+} signal in a large variety of cell types, especially (but not exclusively) in cells of hematopoetic origin. However, in several cell types Ca^{2+}-mobilizing receptors activate PLC in a U-73122-independent manner, but many of these findings have not been published and are known only through personal communications. Part of the problem is technical, as some batches of U-73122 are not as potent as others. More importantly, the DMSO stock solution of U-73122 needs sonication before each use to express its full effectiveness. Nevertheless, even in the same hands, not all GPCR-mediated PLC activation is inhibited by U-73122 (e.g.[122]). This, together with the fact that purified PLC enzymes are not inhibited by the compound in *in vitro* PLC assays, suggests that U-73122 may interfere with the activation process of PLC (or of certain PLC isozymes) rather than with all PLC-s regardless of the type of activation.

U-73122 has been shown to affect several cellular processes apparently independent of its effect on PLC, and many of these effects are also not mimicked by U-73343. One of the best-documented of these is the inhibition of the G_i-mediated negative regulation of cAMP production [123,124]. But other events such as Ca^{2+} influx [125], PMA-induced platelet aggregation [126], or PLD activation [127] have been reported as being sensitive to U-73122 but not U-73343. An additional and important effect of U-73122 on secretion has been reported in the concentration range (5-10 μM) that is usually required to fully inhibit PLC activation. In this concentration range, the compound was found to stimulate the secretion of LH in pituitary gonadotrophs [128], and the secretion of prolactin in GH3 cells [129]. This stimulatory effect of U-73122 on secretion

did not require the presence of external Ca^{2+} and could indicate an additional target, such as a small GTP-binding protein. The possibility of U-73122 affecting G-protein functions is supported by the fact that both PLC activation in hematopoetic cells, and the G_i-mediated inhibition of adenylyl cyclase, are also inhibited by pertussis-toxin treatment, which is known to be mediated by ADP-ribosylation of $G\alpha_i$. Although U-73122 has also been reported to inhibit PLCγ-activation in several cell types (and $PLC\gamma_1$ is not considered as a G protein-regulated enzyme, see above), it should be noted that EGF-mediated activation of PLCγ has been shown to be sensitive to pertussis toxin treatment in liver [130].

In general, U-73122 has been a very valuable tool in analyzing cellular responses linked to PLC activation. Nevertheless, the exact mechanism of its action should be further analyzed, and such analysis could reveal significant new details about the activation mechanisms of the various PLC isoforms in various cell types.

Additional inhibitors of PLC have been reported [131,132], but little data is available to assess their usefulness either as research tools or therapeutic agents.

IV. $Ins(1,4,5)P_3$ ACTION AND METABOLISM

Inositol 1,4,5-trisphosphate (**16**) is the second messenger that links receptor-mediated PLC activation to Ca^{2+} signaling [133]. $Ins(1,4,5)P_3$ acts on specific receptors that are located mainly in the endoplasmic reticulum but also in other intracellular membranes including the nuclear envelope. The receptor is a large protein of 260-300 kDa in size that forms a tetramer and functions as a Ca^{2+} channel. After $Ins(1,4,5)P_3$ binding, Ca^{2+} moves through the $Ins(1,4,5)P_3$ receptor-channel along its concentration gradient from the intracellular stores to the cytosol. Several forms of the $Ins(1,4,5)P_3$ receptor have been cloned (type-1, type-2 and type-3), which show a great deal of sequence homology and have a similar domain structure. The type-I $Ins(1,4,5)P_3$-receptor has several splice variants, some of which are only expressed in neurons, while others only in peripheral tissues. It is generally agreed that most tissues probably contain more than one form of the receptor, and these can even form heterotetramers (see [134] for a recent comprehensive review).

Structurally, $Ins(1,4,5)P_3$ receptors contain an N-terminal $Ins(1,4,5)P_3$-binding domain that binds the ligand even if expressed separately from the rest of the receptor-channel. The C-terminal part of the molecule contains 6 trans-membrane domains and functions as the channel domain. The region between the $Ins(1,4,5)P_3$-binding and the channel domains is the regulatory domain, which contains phosphorylation sites as well as binding sites for other regulators, such as ATP, Ca^{2+}, calmodulin or immunophilins.

IV. 1. $Ins(1,4,5)P_3$-induced Calcium Release

In addition to the $Ins(1,4,5)P_3$-induced Ca^{2+} release, the most unique feature of the $Ins(1,4,5)P_3$ receptor is its Ca^{2+} induced Ca^{2+} release (CICR) property. In this respect the receptor is similar to its structural relative, the ryanodine receptor-channel, found in heart and striated muscle. This feature of the $Ins(1,4,5)P_3$ receptor is caused by a positive effect of Ca^{2+} at the low concentration range (above resting cytosolic Ca^{2+}) on channel opening, while high Ca^{2+} levels (μM range) cause inactivation [135]. Evidence suggests that the Ca^{2+}-dependent inactivation is not present in the type-2 and type-3 $Ins(1,4,5)P_3$ receptors, but these receptors also possess the Ca^{2+}-induced Ca^{2+} release property [136]. According to current models, $Ins(1,4,5)P_3$ determines the threshold level of Ca^{2+} at which $Ins(1,4,5)P_3$ channels open, and it is understood that Ca^{2+} increases without $Ins(1,4,5)P_3$ are not sufficient to activate channel opening. On the other hand, once the channels are activated, $Ins(1,4,5)P_3$ concentrations do not necessarily determine the amount of Ca^{2+} that is released, which is determined mostly by the filling-state of the Ca^{2+} pools and the inactivation of the channel by Ca^{2+} and $Ins(1,4,5)P_3$.

These properties of the $Ins(1,4,5)P_3$ receptor-channel explain the behavior of Ca^{2+} oscillations in cells in which oscillations are driven primarily by

Ca^{2+} release from Ins(1,4,5)P_3-sensitive Ca^{2+} stores [137]. (In many cells Ca^{2+} oscillations are primarily driven by activation and inactivation of Ca^{2+} and K^+-channels of the plasma membrane that serve as pacemakers for the Ca^{2+} release mechanism). In the former case, Ins(1,4,5)P_3 concentrations also determine the lag-time between stimulation and the first Ca^{2+} transient, as well as the frequency of Ca^{2+} oscillations, but only affect the amplitude of Ca^{2+} transients when the time for Ca^{2+} store-refilling becomes a limiting factor.

IV. 2. Agonist-induced Calcium Influx

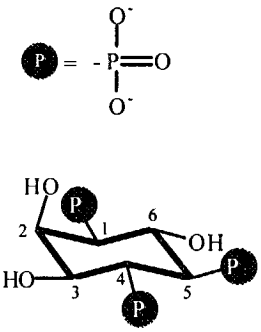
Activation of Ca^{2+} mobilizing receptors usually also increases Ca^{2+} influx from the external medium to the cell interior. This increased Ca^{2+} entry has also been linked to Ins(1,4,5)P_3, not as a direct effect of the messenger but via the emptying of intracellular Ca^{2+} stores. The concept of 'capacitative Ca^{2+} entry' or store-operated Ca^{2+} influx (SOC) was introduced when Ca^{2+} influx was found to be increased even in the absence of increased Ins(1,4,5)P_3 levels after emptying of the intracellular, Ins(1,4,5)P_3-sensitive Ca^{2+} stores [138]. Thapsigargin, for example, which inhibits the Ca^{2+} ATPase that pumps Ca^{2+} and fills the Ca^{2+} stores, leads to Ca^{2+} pool depletion (through a leak Ca^{2+} current), and activates Ca^{2+} influx without an apparent increase in Ins(1,4,5)P_3 levels [139]. The molecular entity that is responsible for Ca^{2+} entry through SOC has not yet been identified, nor has the mechanism by which the filling-state of the Ca^{2+} pools communicates this information to the plasma membrane [140]. One remarkable set of observations may be important in this regard. A mechanism requiring GTP hydrolysis has been found to determine the amount of Ca^{2+} that can be released by Ins(1,4,5)P_3 in microsomal preparations as well as in permeabilized cells [141-143]. Moreover, SOC has been shown to be inhibited by non-hydrolyzable GTP analogs [144,145], and it was postulated that perhaps a GTP-sensitive membrane fusion event is needed to allow for the enhanced Ca^{2+} influx to take place in the presence of Ins(1,4,5)P_3 [146-148]. It is also noteworthy that inositol 1,3,4,5-tetrakisphosphate (Ins(1,3,4,5)P_4) which is produced from Ins(1,4,5)P_3 has also been found to be a regulator of Ca^{2+} influx in conjunction with Ins(1,4,5)P_3 [149]. The Ins(1,3,4,5)P_4 receptor, on the other hand, was identified as a GTP-ase activating protein (GAP) for the small GTP binding proteins Rap and Ras [150].

Recent data also indicate a physical interaction between the N-terminal Ins(1,4,5)P_3 binding domain of Ins(1,4,5)P_3 receptors and certain forms of the mammalian Trp-channels. A model assuming a physical interaction between Ins(1,4,5)P_3 receptors and the elusive Ca^{2+} entry channels had already been proposed earlier [151]. The Trp channel was first identified in the fruit fly, *Drosophila melanogaster*, as a putative Ca^{2+} channel responsible for the sustained depolarizing current that is observed after photo-stimulation of the eye [152]. Since the main signal transduction pathway in photo-receptor signaling in invertebrates is the PLC-mediated production of Ins(1,4,5)P_3, it has been suggested that Trp is the Ca^{2+} channel that is regulated by Ins(1,4,5)P_3, and might be identical to the elusive store-operated Ca^{2+} channel. Indeed, mammalian homologues of Trp have been cloned (Trp1-7), and when expressed in cells cause an altered Ca^{2+} influx response after stimulation. Yet, while Ca^{2+} entry can be increased by agonists or Ins(1,4,5)P_3 in cells expressing the various Trp proteins, their response to store depletion (especially for Trp3 and 6) is more ambiguous [153,154]. Also, Trp3 and Trp6 can be regulated by diacylglycerol analogs independent of PKC activation or store depletion [155]. Moreover, Trp channels do not have the same ion selectivity that was described for SOC, or for I_{CRAC}, the whole cell current that can be detected in patch clamp studies after Ca^{2+} store depletion [156]. While more data are needed to clarify the relationships between SOC, I_{CRAC}, Trp channels and Ins(1,4,5)P_3-regulated Ca^{2+} entry, the recently described physical connection between Ins(1,4,5)P_3 channels and the Trp3 protein [157-159] may represent an important link between Ins(1,4,5)P_3-induced Ca^{2+} release and regulation of Ca^{2+} influx.

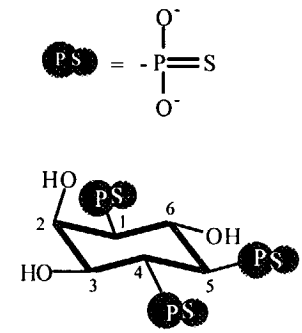
IV.3. Pharmacology of the Ins(1,4,5)P_3 Receptor

The pharmacology of the Ins(1,4,5)P_3 receptor has been recently covered in a comprehensive review [160], and only a short summary will be presented here. Most data have been obtained with the most abundant type-1 receptor, but it appears that type-2 and –3 receptors do not differ enough from the type-I form so that they could be differentially modulated. Initial attempts characterizing the binding and Ca^{2+} mobilizing properties of inositol phosphate analogs already revealed a strict stereospecific requirement, as the L-form of Ins(1,4,5)P_3 was found 1000-fold less potent than the natural D-isomer. These studies also revealed that either addition or removal of a phosphate from the Ins(1,4,5)P_3 molecule greatly decreases both the binding affinity and the Ca^{2+} release potency of the analogs. The presence of phosphates in the 4-and 5-positions are of crucial importance for potency, while switching the phosphate group from position 1 to position 2, still yields an active molecule, although about 10 times less potent than Ins(1,4,5)P_3 [161](Fig. **8**). This isomer, **Ins(2,4,5)P_3 (17)**, has been widely used because of its limited metabolism by both the 3-kinase and the 5-phosphatase enzymes (see below). Additional metabolically stable analogs bearing phosphorothioate groups instead of the phosphate groups **[Ins(1,4,5)PS_3] (18)** also showed relatively good affinities and calcium-mobilizing abilities [162], suggesting that the slightly bigger phosphorothioate groups are tolerated within the Ins(1,4,5)P_3 binding pocket of the receptor.

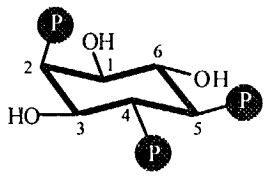
A crucial question to be answered in Ins(1,4,5)P_3 receptor pharmacology is the nature of the conformational change that transmits ligand binding information to channel opening. Good inhibitors should bind to the receptor without evoking the conformational change, i.e. should have high affinity with zero efficacy. Much less is known about the structural features of analogs that determine their efficacy. The phosphate group at position 1 seems to be one determinant of the agonistic property of Ins(1,4,5)P_3 [163], but modifications of the 3-hydroxyl positions also resulted in a decreased efficacy [164]. One of the best partial agonist described so far is the 3-fluorinated 4,5-phosphorothioate analogue of Ins(1,4,5)P_3 **[3F-Ins(1)P(4,5)PS_3] (19)**, which displays a relatively high affinity with modest intrinsic activity [163]. Highly potent, non-inositol phosphate agonists have been recently isolated from fungal cultures and were structurally



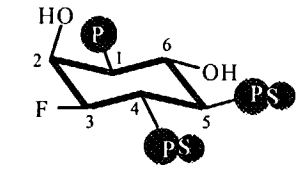
inositol 1,4,5-trisphosphate (**16**)



inositol 1,4,5-trisphosphorothioate (**17**)

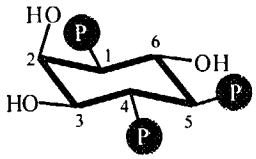


inositol 2,4,5-trisphosphate (**17**)

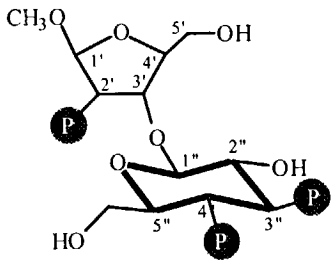


D-3-fluoro-myo-inositol 1-phosphate - 4,5-bisphosphorothioate (**19**)

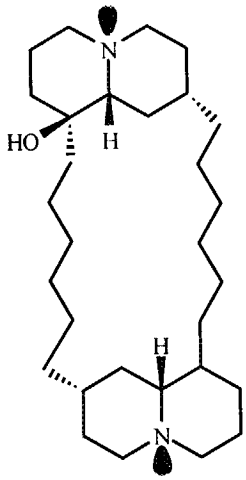
Fig. (8).



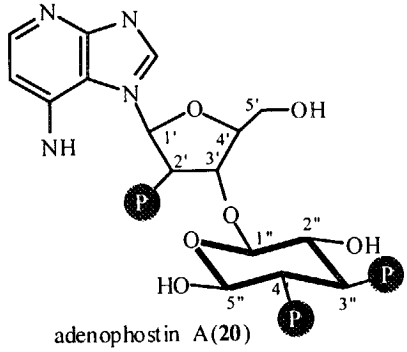
inositol 1,4,5-trisphosphate (**16**)



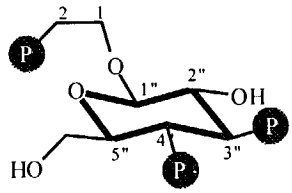
methyl-3-O-(α-D-glucopyranosyl)-β-D-ribofuranoside 2,3',4'-trisphosphate (**21**)



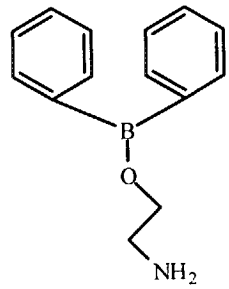
xestospongin C (**23**)



adenophostin A(**20**)



2-hydroxyethyl-α-D-glucopyranoside-2,3',4'-trisphosphate (**22**)



2-aminoethoxydiphenyl borate (2-ABP) (**24**)

Fig. (9).

identified as **adenophostins** [165] (Fig. **9**). The structures of these compounds are completely different from that of Ins(1,4,5)P_3, but molecular modeling studies showed that the distribution of their phosphate groups can effectively mimic the natural ligand in its interaction with the receptor [165]. Several analogs of **adenophostin A (20)** have been synthesized to provide additional structural information about the optimal positions of the various phosphate groups for binding and intrinsic activity of an analog. For example, **methyl-3-O-(α-D-glucopyranosyl)-β-ribofuranoside 2,3',4'-trisphosphate (21), or 2-hydroxyethyl-α-D-glucopyranoside-2,3',4'-trisphosphate** (**22**), both appear to mimic the **adenophostin A** structure in activating the Ins(1,4,5)P_3 receptors, although about 10-fold less potently [166-168]. Because of their metabolic resistance and high Ca^{2+}-releasing potency, these compounds have a great potential in studying Ins(1,4,5)P_3 receptor function, once their cell-permeable analogs become available for use in intact cells.

No good antagonists of the Ins(1,4,5)P_3 receptors have been found in spite of many efforts using the inositol (whether *myo-*, *scillo-*, or *chiro-*) or cyclohexane backbone to create inhibitory analogs. Most investigators have used **heparin** to anatgonize Ins(1,4,5)P_3 action on the receptor, and

certainly heparin can block Ins(1,4,5)P_3-induced Ca^{2+} release in cell-free systems or when injected into cells [163]. However, heparin is a very non-selective tool and will bind to a large number of proteins with basic residues to inhibit their function. **Decavanadate**, has also been shown to block Ins(1,4,5)P_3-induced Ca^{2+} release, but it also suffers from non-selectivity and can be converted to other forms of vanadate with a completely new spectrum of targets [169]. More recently, two new antagonists of Ins(1,4,5)P_3 receptor have been described (Fig. **9**). **Xestospongins (23)** have been isolated from the Australian sponges of *Xestospongia*, and were shown to inhibit Ins(1,4,5)P_3-induced Ca^{2+} release in cell-free system (IC_{50}~0.3 μM) as well as in cells, since they are cell permeable [170]. Xestospongins also block ryanodine receptors, but only at 30 times higher concentrations. They do not compete with Ins(1,4,5)P_3 binding and probably inhibit the channel by interacting with its pore or by allosterically preventing the conformational change that results in channel opening [170]. Importantly, Xestospongin C was also found to inhibit the endoplasmic reticulum Ca^{2+} pump with equal potency [171], raising the question whether it affects the Ca^{2+} binding site(s) of certain pumps and channels. The other compound, **2-aminoethoxyldiphenyl borate (2-APB) (24)** was found to inhibit Ins(1,4,5)P_3 action (IC_{50}: 42 μM) in platelets and neutrophils, and unlike xestospongins, does not inhibit the ryanodine channel [172].

V. METABOLISM OF Ins(1,4,5)P_3

Once produced, Ins(1,4,5)P_3 is metabolized extremely rapidly within the cell. Its major route of elimination is by dephosphorylation at position 5 to produce Ins(1,4)P_2, which, in turn, is further dephosphorylated at position 1 to yield Ins(4)P. Some of the Ins(1,4)P_2 can be produced by direct hydrolysis of PtdIns(4)P by PLC, but the contribution of this pathway to Ins(1,4)P_2 production is difficult to quantitate. Similarly, Ins(1)P, can be formed from direct hydrolysis of PtdIns, but it is also produced from Ins(1,4)P_2, as a minor route of Ins(1,4)P_2 dephosphorylation.

V.1. Ins(1,4,5)P_3 3-KINASE

An alternative route of Ins(1,4,5)P_3 elimination is its phosphorylation at the 3-position by an Ins(1,4,5)P_3 3-kinase (not to be confused with PI 3-kinases, see above) to form Ins(1,3,4,5)P_4 [12]. The conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 is stimulated by Ca^{2+}-calmodulin [173] and Ins(1,3,4,5)P_4 has been proposed to act in concert with Ins(1,4,5)P_3 to regulate Ca^{2+} entry (see above, and also[13]. Three isoforms (A,B and C) of 3-kinases that catalyze this reaction have been identified by molecular cloning from human[174,175](and GeneBank accession #D38169). The conversion of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4 adds a number of inositol phosphates as metabolic intermediates of Ins(1,3,4,5)P_4 metabolism, Ins(1,3,4)P_3 being the first to be identified. This Ins(1,4,5)P_3 isomer is inactive as far as calcium mobilization is concerned, but its level far exceeds that of Ins(1,4,5)P_3 after stimulation, especially if cells are treated with Li^+ to enhance increases in InsP levels (see below). This makes it necessary to separate the two isomers by HPLC when analyzing Ins(1,4,5)P_3 changes from radio-labeled cells.

Ins(1,3,4)P_3 is dephosphorylated via two different routes, via Ins(3,4)P_2 and Ins(1,3)P_2. The relative contributions of the two pathways varies from cell to cell, the former being usually more prominent. Analysis of these different metabolic intermediates may seem to be irrelevant, since they are believed to be biologically inactive. However, it is quite possible that some of the metabolites (or others that co-elute with them on HPLC) are, in fact, part of a synthetic route(s) by which inositol higher phosphates, such as InsP_5 and InsP_6 are synthesized (see below). For example, Ins(3)P, which is an intermediate of Ins(1,3,4)P_3 metabolism, is also the first step in InsP_6 synthesis in *Dyctostelium* [176], and cannot be separated by HPLC from its enantiomer, Ins(1)P, which can be also produced in alternative ways (see above).

The enzymes that metabolize Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 have been purified and cloned and, not surprisingly, show a similar complexity as the

rest of the enzymes that contribute to inositide metabolism.

V. 2. Inositol Phosphate and Inositol Lipid Phosphatases

Ins(1,4,5)P_3 5-phosphatases

This enzymatic activity was first described in liver membranes [177], and was later purified from platelet membranes as two activities, termed type-I and type-II [178,179]. After cloning of the first 5-phosphatase (the type II enzyme from platelets), several 5-phosphatases have been identified and today there are eight mammalian genes encoding for distinct 5-phosphatases that show extensive homologies in two of their signature motifs. Some of these enzymes are involved in the dephosphorylation of inositol lipids rather than inositol phosphates, and substrate preference serves the basis for their classification (see [180] for a recent review on inositol phosphate and inositide phosphatases).

Group I 5-phosphatases are the enzymes that dephosphorylate only water-soluble Ins(1,4,5)P_3 and Ins(1,3,4,5)P_4 and hence, regulate Ca^{2+} signaling. The 43 kDa platelet type I enzyme belongs to this group and its membrane attachment is provided by isoprenylation. This enzyme has been shown to be activated by PKC. This effect apparently mediated by pleckstrin, which strongly associates with the protein and is the major PKC substrate in platelets.

Group II 5-phosphatases, can dephosphorylate the lipids, PtdIns(4,5)P_2 and PtdIns(3,4,5)P_3, in addition to the soluble inositol phosphates. The 75 kDa type II platelet enzyme and the OCRL protein belong to this group of enzymes, and show 51% identity within a 744 stretch of their amino acid sequence. The mutated OCRL-1 gene is responsible for the X-linked human disease, oculocerebrorenal syndrome of Lowe [181], which is characterized by renal tubular acidosis, mental retardation, early development of cataracts, retinal degeneration and renal failure. The OCRL protein is believed to be associated primarily with the trans-Golgi network [182], although it was also shown to be present in lysosomal membranes. The platelet type II enzyme, on the other hand, is associated with the plasma membrane and mitochondria through isoprenylation. It is not clear at present how a defect in OCRL protein function leads to the development of the symptoms of Lowe's disease, but it has been proposed to cause a defect in lysosomal sorting. However, mice lacking either the OCRL or type II phosphatase do not develop symptoms similar to Lowe's syndrome, but elimination of both enzymes is an embryonic lethal. It is very likely that the primary substrate of both of these enzymes is the inositol lipid in the target membranes.

Additional members of the group II 5-phosphatases are synaptojanin and synaptojanin 2, two enzymes that are also believed to hydrolyze inositol lipids rather than inositol phosphates. These proteins have been implicated in synaptic vesicle trafficking [183] and associate with other proteins, such as amphyphysin and dynamin, both of which are known to be important for synaptic vesicle recycling. In addition to the 5-phosphatase signature motifs, synaptojanin contains a C-terminal SH3 domain, and both enzymes contain SacI domains named after the yeast protein, SacI [184].

Group III 5-phosphatases only hydrolyze substrates that contain a phosphate group at position 3 of the inositol ring, i.e. Ins(1,3,4,5)P_4 or PtdIns(3,4,5)P_3. Two enzymes belong here, SHIP and SHIP-2, the former being expressed only in cells of hematopoietic origin and the latter being more widely distributed. These enzymes have splice variants and also could undergo C-terminal proteolytic truncations, adding to the variety of molecular size in which they appear (110-145 kDa) (see [185]). They all have an N-terminal SH2 domain through which they bind to activated cytokine or growth factor receptors. They also contain proline-rich sequences in their C-termini. Because of their ability to hydrolyze PtdIns(3,4,5)P_3, a lipid that is produced by PI 3-kinases and is not hydrolyzed by PLC, SHIP and SHIP2 could serve as important negative regulators to terminate activation of PtdIns(3,4,5)P_3-

dependent activation pathways. Targeted elimination of SHIP, indeed, leads to enhanced cytokine signaling and proliferation of myeloid cells [186].

Group IV 5-phosphatases are 70-75 kDa enzymes that also hydrolyze PtdIns(3,4,5)P_3, and to a lesser degree PtdIns(4,5)P_2 [187]. These proteins are very rich in prolines, especially in their N-termini. In one study, the enzyme was found to localize to Golgi membranes [188], while a similar enzyme isolated from the rat seems to localize mostly to membrane ruffles [189]. Little is known about the importance of these novel proteins.

Inositol Polyphosphate 1-Phosphatase and Inositol Monophosphatase

There is only one known inositol polyphosphate 1-phosphatase, which hydrolyzes the 1-phosphate of its substrates, Ins(1,4)P_2 and Ins(1,3,4)P_3, and this enzyme does not dephosphorylate inositol lipids [190]. A functional relative of this protein is the enzyme, inositol monophosphatase [191], that dephosphorylates both Ins(4)P and Ins(1)P. However, there is little sequence similarity between the two enzymes apart from their active sites. Inositol monophosphatase works as a homodimer containing two 30 kDa subunits and, unlike many other phosphatases, catalyses the direct hydrolysis of its substrate without forming an E-P intermediate. This enzyme can hydrolyze phosphates from other positions of the inositol ring, except the axial 2-position so Ins(2)P is not a substrate of the enzyme. Both the inositol monophosphatase and the inositol polyphosphate 1-phosphatase can be inhibited by lithium ions, a property that drew attention to their function(s) and importance (see below). Together with the group I 5-phosphatase, these are the enzymes that are responsible for effective recycling of the inositol phosphates into PtdIns resynthesis in agonist-stimulated cells.

PTEN

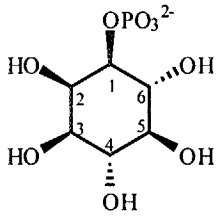
The tumor supressor gene product, PTEN, has been recently shown to be an inositol lipid phosphatase which hydrolyses the 3-phosphate group, although it has relatively broad substrate specificity. PTEN could exert its tumor supressor action by hydrolyzing PtdIns(3,4,5)P_3, acting similarly to SHIP by antagonizing the effects of growth factors that increase the level of this phosphoinositide (see [192]). However, PTEN also possesses protein phosphatase activity that has been claimed to be important in its tumor supressor action. Recent studies on PTEN-deficient embryonic stem cells showed elevated PtdIns(3,4,5)P_3 levels and increased PKB/Akt activity [193]. Moreover, embryonic fibroblasts derived from PTEN-deficient mice (which is an embryonic lethal) display significantly increased Rac1 and Cdc42 GTPase activities [194], and these small G nucleotide binding proteins are known targets of PtdIns(3,4,5)P_3. Altogether there is a strong case for a connection between PTEN and PtdIns(3,4,5)P_3-regulated, processes and targeting PTEN function with inhibitors is probably a top priority in several laboratories. The recently published crystal structure of the protein [195] should greatly aid these efforts.

V.3. Inhibitors of Inositol Phosphate Metabolism

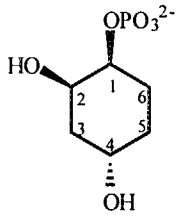
Inhibition of Ins(1,4,5)P_3 metabolism has been mostly sought as a tool to better understand the role of this messenger and its phosphorylated form, Ins(1,3,4,5)P_4 in calcium signaling. However, even before Ins(1,4,5)P_3 was recognized as a Ca^{2+}-mobilizing intracellular messenger, inositol phosphate phosphatase enzymes became extremely important candidates as targets in the treatment of manic-depressive disorders. Lithium has been used as one of the most effective treatments of bipolar mood disorders, but the mechanism of its beneficial effects was initially unknown. Allison and Stewart then noted that Li^+ administration decreases the level of free inositol in rat brain together with increased level of inositol 1-phosphate [196], and subsequently it was demonstrated that Li^+ at its therapeutically effective dose (1 mM) inhibits inositol monophosphatase activity (K_i: 0.8 mM) [197]. It was also noted, that the inhibition was of a

peculiar uncompetitive nature in that the higher concentration of substrate was used, the higher potency Li^+ displayed [198]. Berridge *et al.* showed that Li^+ can be used to amplify agonist-induced inositol phosphate responses, and noted that inositol phosphate accumulation became progressively larger at high, supramaximal concentrations of the agonists [199]. These observations led to the proposal that the beneficial effects of lithium in the treatment of manic-depressive disease arise from its ability to selectively affect groups of hyperactive neurons without impairing the function of those with normal activity [199]. Also, because of the limited availability of *myo*-inositol in the brain (it does not cross the blood-brain barrier [26]), *myo*-inositol depletion affects the brain significantly more than peripheral tissues. This theory became the most popular explanation for the therapeutic effects of lithium, but the cation could affect cellular functions in a number of alternative ways and there is still uncertainty concerning its mechanism of action. Recently, important observations obtained in mutants of *Drosophila melanogaster* in which the inositol polyphospate 1-phosphatase levels (see above) are severely impaired, further substantiated the role of this enzyme in CNS function. Mutant flies show a "shaker" phenotype and a severe defect in synaptic transmission. Similar phenotypic changes can be observed in wild-type flies after treatment with lithium, but Li^+ treatment has no further effect on flies that are deficient in inositol polyphosphate 1-phosphatase [200]. Moreover, of two human inositol monophosphatase genes, one (IMPA2) is localized to a putative susceptibility region for bipolar disorder on chromosome 18p11.2 [201] and showes significant polymorphism in a sample of Norwegian bipolar patients [202].

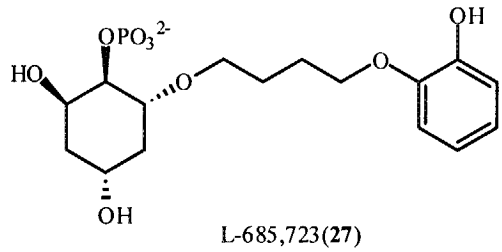
All of these data indicated the need to find alternative inhibitors of inositol monophosphatase, especially since the therapeutically effective (1 mM) and toxic levels (2 mM) of Li^+ are extremely close. These efforts have been discussed in a recent review [203], so only a summary of these will be presented below. Studies with **inositol phosphate-based derivatives** concluded that the OH-groups at positions 2 and 4 are important for binding while that at position 6 is implicated in catalysis. Accordingly, derivatives missing -OH in positions 3 and 5 (such as **26**), and in which the 6-OH was replaced by various groups (as in **27**) were found to be inhibitors with good affinity, the most promising bearing lipophilic substitutions [204] (Fig. **10**). However, these compounds had poor potencies *in vivo*, probably because of their metabolic instability and susceptibility to phosphatases [205]. To create phosphatase-stable analogues, the phosphate group was replaced by



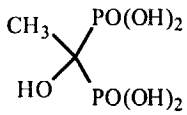
myo-inositol-1-phosphate (**25**)



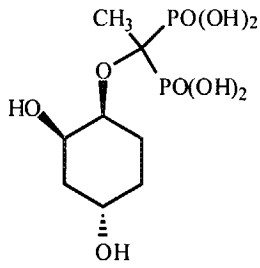
2,4-dihydroxy-inositol-1-phosphate (**26**)



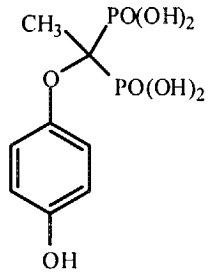
L-685,723(**27**)



1-hydroxyethylidene-1,1-bisphosphonic acid (**28**)



L-687,588 (**29**)



L-690,330 (**30**)

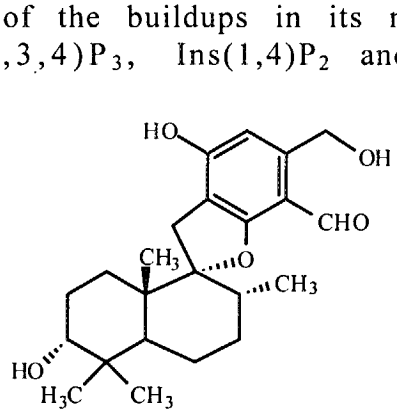
Fig. (10).

bisphosphonates (29), which created a new set of inositol monophosphatase inhibitors. The best of this series were **hydroxymethylene bisphosphonic acid (28)** derivatives (such as **30**) in which an aromatic ring replaced the inositol ring of the original substrate, Ins(1)P (L-690,330 IC_{50}: 0.3 μM) [205]. After optimization and adding pivaloyloxy-methyl esters (that will be hydrolyzed by intracellular esterases) to increase cell permeability, promising compounds were created that reproduced the effects of Li^+ in brain slices. Unfortunately, these were not effective in whole animals probably due to solubility problems at the sites of injection [206].

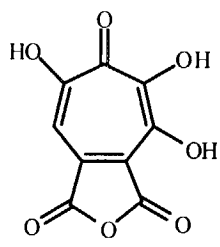
As with the other enzymes, a search for naturally occuring inhibitors of inositol monophosphatase has also been performed (Fig. **11**). This identified a non-competitive inhibitor **sesquiterpene (31)** (ATCC 20928, L-671,776, IC_{50}:70-200 μM) [207], and the competitive inhibitor **puberulonic acid (32)** (IC_{50}: 10 μM) [208]. Interestingly, none of these compounds contains a phosphate group. Several **tropolone** derivatives have been tested to determine the structural basis of their inhibitory effect, but none of these was significantly better than the parent compound, **3,7-dihidroxytropolone (33)** (IC_{50}: 10 μM) [209]. It is yet to be seen whether this direction of research could yield inhibitors that match the effects of Li^+ in *in vivo* experiments.

It is important to point out that addition of lithium does not lead to the accumulation of the calcium-mobilizing messenger, $Ins(1,4,5)P_3$, in spite of the buildups in its metabolites, $Ins(1,3,4)P_3$, $Ins(1,4)P_2$ and the Ins-monophosphates. In fact, the acute effect of Li^+ on $Ins(1,4,5)P_3$ levels is inhibitory, probably due to a feed-back inhibition of PLC-s from the accumulating products. These inhibitory effects can be observed even before inositol-depletion impacts $PtdIns(4,5)P_2$ levels, since the latter can be maintained even when the PtdIns pool is decreasing due to dropping *myo*-inositol levels [210,211].

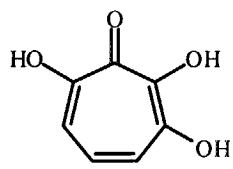
To increase $Ins(1,4,5)P_3$ levels, one needs to inhibit the 5-phosphatase enzyme(s), but good inhibitors of this enzyme have not been reported so far. Several analogs of $Ins(1,4,5)P_3$, either deoxygenated [212] or fluorinated [213] have been tested as inhibitors of 5-phosphatase, and although some compounds were found to be potent inhibitors of the enzyme, none can be used in intact cells. Similarly, $Ins(1,4,5)P_3$ 3-kinase inhibitors would be very useful research tools to better understand the signaling role(s) of $Ins(1,3,4,5)P_4$. Since this enzyme is activated by Ca^{2+} - calmodulin, calmodulin inhibitors seemed as logical tools to inhibit this conversion. However, our experience is that many calmodulin inhibitors inhibit $Ins(1,4,5)P_3$ formation and $Ins(1,4,5)P_3$-induced Ca^{2+} release, before they noticably change the metabolic route of $Ins(1,4,5)P_3$ metabolism. The cytostatic drug, **adriamycin** has been found to inhibit $Ins(1,4,5)P_3$ 3-kinase and the production of $Ins(1,3,4,5)P_4$ in T cells [214], but the specificity of this effect and the mechanism by which the drug affects the enzyme is unknown. We have shown previously, that incubating adrenal glomerulosa cells in Sr^{2+} instead of Ca^{2+}, had relatively small impact on receptor-mediated PLC activation, but greatly diminished the conversion



L-671,776 (**31**)



puberulonic acid (**32**)



3,7-dyhydroxytropolone (**33**)

Fig. (11).

of Ins(1,4,5)P_3 to Ins(1,3,4,5)P_4, thereby increasing Ins(1,4,5)P_3 levels [215]. Although this manipulation has no therapeutic value, it may be a simple way of altering Ins(1,4,5)P_3 metabolism in cultured cells.

Inhibitors of the phosphatases that dephosphorylate inositol lipids as opposed to inositol phosphates have not yet been identified. Although such inhibitors would be very useful research tools, it is not clear at present whether they could be of therapeutic value, since they usually serve as terminators, or negative regulators of intracellular signals.

VI. HIGHER PHOSPHORYLATED INOSITOLS

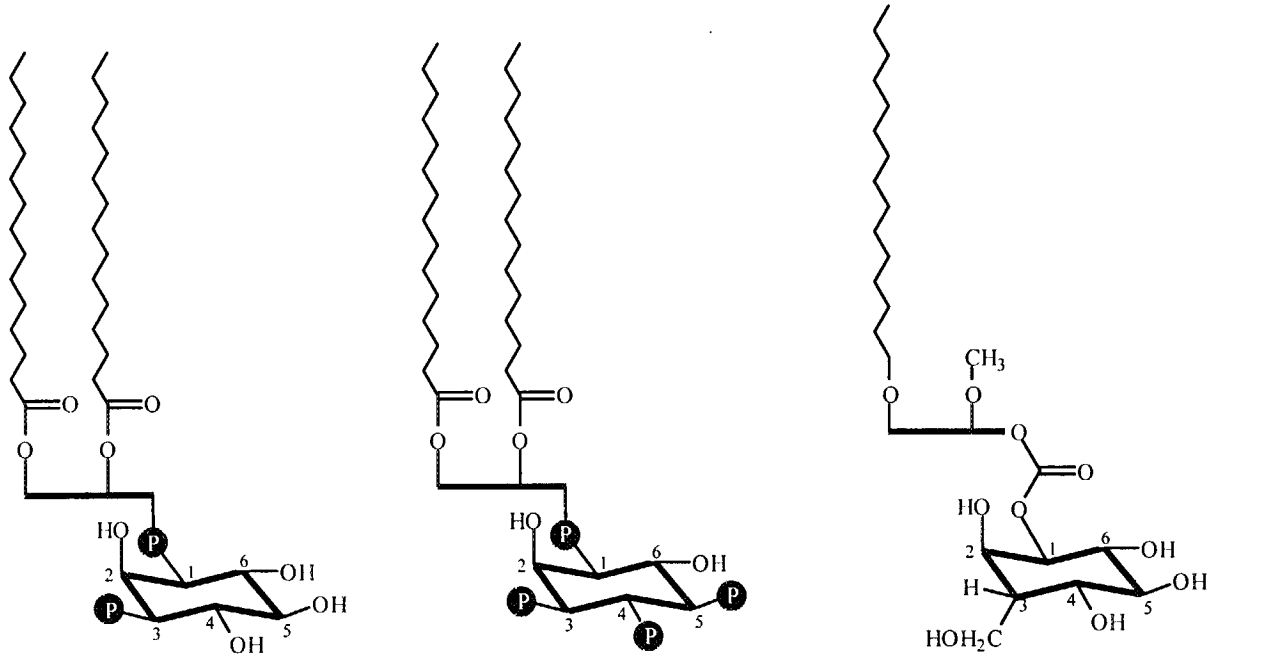
As soon as HPLC was applied to analyze inositol phosphates from radio-labeled cells, the presence of higher inositol phosphates was noted [14,216]. These included three additional $InsP_4$ isomers [Ins(1,3,4,6)P_4, Ins(3,4,5,6)P_4 and its enantiomer, Ins(1,4,5,6)P_4] as well as $InsP_5$ and $InsP_6$. Moreover, even more phosphorylated forms of inositol phosphates have been detected, containing pyrophosphates on their inositol ring [16,217]. Heroic efforts have been mobilized to determine the specific activities of the individual phosphates on the inositol ring of inositol polyphosphates in non-equilibrium conditions in order to obtain information on the sequence of their phosphorylation [218]. However, because of the complexity of InsP formation and the probable existence of multiple metabolic pools, no unequivocal answers were obtained concerning a synthetic pathway in mammalian cells.

The only link that appeared to connect the inositol higher phosphates to the 'classical' Ins(1,4,5)P_3, in mammalian cells was the pathway that converts Ins(1,3,4)P_3 to Ins(1,3,4,6)P_4 [219,220] with subsequent production of $InsP_5$. The other $InsP_4$ isomer, Ins(3,4,5,6)P_4, was found to be produced from $InsP_5$ in cell free systems using cytosol [221,222]. However, isotope studies showed that the labeling of the highly phosphorylated inositols was very slow, and changed only after prolonged agonist stimulation [223]. It was also observed, that labeling of $InsP_5$ and Ins(3,4,5,6)P_4/Ins(1,4,5,6)P_4] correlated with the growth-rate of cells, which explained why their labeling was very high in transformed cells [224,225]. Subsequent studies revealed that the labeling of these higher inositol phosphates is increased in the S-phase of the cell cycle [224], a finding that may be more relevant in light of recent advances in this field. A regulatory role of some of these compounds, such as Ins(3,4,5,6)P_4 to affect calcium-dependent chloride currents has been shown [226], and an effort to isolate $InsP_5$/$InsP_6$ binding proteins identified the clathrin adaptor, AP-2 as a receptor [227]. Generally, the function of these compounds has remained obscure, and although the Ins(1,3,4)P_3 5/6-kinase have been cloned [228], metabolic studies could not substantiate this pathway as the major route leading to $InsP_5$ and $InsP_6$ synthesis.

More recent studies in yeast shed new lights on the possible functions of inositol higher phosphates. Two genes have been identified in *Saccharomyces cerevisiae* that play a critical role in mRNA export from the nucleus [229]. One of these enzymes, Ipk2, synthesizes Ins(1,4,5,6)P_4 from Ins(1,4,5)P_3, and is identical to Arg82, a regulator of the transcriptional complex, ArgR-Mcm1 [230]. The other enzyme, Ipk1, is an Ins(1,3,4,5,6)P_5 2-kinase, that produces $InsP_6$ or phytic acid [231]. It is not yet understood how these inositol phosphates work in the yeast or in mammalian cells, but these studies represent breakthrough in pointing to the importance of these compounds. Extension of these studies certainly will soon teach us more about the functions of inositol higher phosphates. In light of the fact that the physiological role of these pathways has remained elusive for so long, it is not surprising that their pharmacological manipulation has not been a top research priority. Hopefully, this will change in the near future.

VII. FUTURE DIRECTIONS

What emerges even from this simplified summary is that very few inhibitors of inositide-based signaling events qualify as specific tools to dissect the importance of any single biochemical



Ptdins(3)P (**34**) PtdIns(3,4,5)P_3 (**35**) 1L-6-(hydroxymethyl)-*chiro*-inositol-2-[(R)-2-O-methyl-3-O-octadecylcarbonate (**36**)

Fig. (12).

step in any given cellular response, let alone be used as therapeutic agents. Inositol lipid kinase inhibitors have been remarkably useful research tools, but even if their specificity can be greatly increased to discriminate between the various classes of PI 3 and 4-kinases, the question remains whether inhibiting the production of PtdIns(3,4,5)P_3 (**35**) may affect the several signaling pathways that use this lipid as its regulator. However, as we learn more about the recruitment and molecular interactions of the various enzymes involved in inositide metabolism with other signaling proteins, we could clearly envision that small molecules can be developed that could inhibit molecular interactions that are critical to the regulation of a particular enzyme. For example, inhibition of the association of the SH2 domain of p85, the adaptor protein of Class Ia PI 3-kinases, with tyrosine-phosphorylated receptors or adapter proteins could selectively inhibit activation of this group of enzymes without affecting PI 3-kinase γ, or the other PI 3-kinases. Similarly, our understanding of the molecular basis of recognition of the various isomers of phosphoinositides has significantly advanced in recent years. Protein modules, such as the pleckstrin-homology (PH)- and FYVE domains, have been identified in important signaling proteins as docking modules with the ability to recognize inositides with remarkable specificity [19,232-234]. These interactions can also be targeted with small molecules, and recent efforts to disrupt these interactions started to appear in the literature. For example, 3-(hydroxymethyl)-bearing phosphati-dylinositol ether lipid analogs, such as (**36**) (Fig. **12**) [235] as well as inositol phosphate isomers [236], have been found to interfere with, PtdIns(3,4,5)P_3-mediated activation of the Akt kinase pathway. Also, it is quite possible that the mechanism of inhibitory action of U73122 on PLC activation is also based on disruption of regulatory contacts. As we learn more about the structural features of the protein-protein and protein-inositide interactions, more efficient methods can be developed to screen for suitable small molecules. These strategies may help find the specificity that is required for selective manipulation of individual pathways and to develop drugs that can be used in clinical therapy.

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FOOTNOTES

[1]: The abbreviations used are: phosphatidylinositol, PtdIns; phosphatidic acid, PtdA; diacylglycerol, DAG; inositol 1,4,5-trisphosphate, Ins(1,4,5)P_3; phospholipase C, PLC; protein kinase C, PKC; store-operated Ca^{2+} entry, SOC;

[2]: conventionally, the older abbreviation of phosphatidylinositol, (PI) is used in the context of PI and PIP kinases, and PtdIns 3-kinase, specifically denotes the mammalian Class III PI 3-kinase. Therefore PI and PIP will be used when referring to the kinases throughout this review.

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